



# Intraordinal phylogenetics of treeshrews (Mammalia: Scandentia) based on evidence from the mitochondrial 12S rRNA gene

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## Abstract

Despite their traditional and continuing prominence in studies of interordinal mammalian phylogenetics, treeshrews (order Scandentia) remain relatively unstudied with respect to their intraordinal relationships. At the same time, significant morphological variation among living treeshrews has been shown to have direct relevance to higher-level interpretations of character state change as reconstructed in traditional interordinal studies, which have often included only a single species of treeshrew. Therefore, the importance of resolving relationships among treeshrews extends well beyond a better understanding of patterns of diversification within the order. A recent review highlighted several shortcomings in published studies of treeshrew phylogenetics based on morphology. Here we present the first investigation of treeshrew phylogenetics based on DNA sequences, utilizing previously published sequences from the mitochondrial 12S rRNA gene and combining them with newly generated sequence data from 15 species. Parsimony, likelihood, and Bayesian analyses all strongly support a sister relationship between *Ptilocercus* and the remaining species, further substantiating its recent elevation to familial status. *Dendrogale* is consistently recovered as the next taxon to diverge, but relationships among the remaining taxa are poorly supported by these data. We provide evidence for a relatively rapid radiation within the genera *Tupaia* and *Urogale*, but limited resolution precludes more than a cursory interpretation of biogeographic patterns.

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## 1. Introduction

Treeshrews (Mammalia: Scandentia) are small-bodied mammals from South and Southeast Asia whose interordinal relationships have been investigated in several morphological and molecular studies (e.g., Bloch and Boyer, 2002; Murphy et al., 2001a; Sargis, in press; Silcox, 2001). Their intraordinal relationships, on the other hand, have received relatively little attention (see review by Olson et al., 2004a). Unfortunately, this has led

those studying placental supraordinal relationships to use a single species of *Tupaia* (e.g., *T. glis*, *T. belangeri*, or *T. tana*) to represent all of Scandentia in such studies (e.g., Beard, 1993; Murphy et al., 2001a). This is particularly problematic because Sargis (2002a) showed that the inclusion of *Ptilocercus*, thought to be the most plesiomorphic living treeshrew (e.g., Emmons, 2000; Le Gros Clark, 1926; Martin, 1990; Sargis, 2000, 2002a,b, 2004, in press; Szalay and Drawhorn, 1980), in such analyses has a significant effect on the results, as its addition changes the topology of the resulting trees (Sargis, in press).

A well-corroborated intraordinal phylogeny is needed to address several issues in treeshrew biology that go beyond phylogenetic relationships and taxonomy, including: (1) morphological and behavioral evolution,

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(2) relationships of fossils, and (3) biogeography of the group. Concerning the first of these topics, Sargis (2000, 2002a,b, 2004) has proposed several hypotheses of scandentian postcranial evolution (see also Szalay and Drawhorn, 1980), and similar hypotheses of craniodental and soft tissue evolution have been proposed as well (e.g., Butler, 1980; Davis, 1938; Le Gros Clark, 1926; Lyon, 1913). All of these hypotheses of morphological evolution can be tested once a well-supported phylogeny is available. The same is true for hypotheses of behavioral evolution, such as the one proposed by Sargis (2001) for the evolution of grasping in treeshrews.

Regarding the second issue, the scandentian fossil record is quite poor (Sargis, 1999, 2004), making it difficult to determine the relationships of fossil treeshrews. These relationships are further confounded by our lack of understanding regarding the phylogeny of living scandentians, so a viable phylogenetic hypothesis of extant treeshrew relationships is necessary to provide a framework within which the phylogeny of fossil taxa can be assessed using morphological evidence.

Finally, the biogeographic history of treeshrews is poorly understood, not because of a lack of understanding of the geologic history of South and Southeast Asia, but because of a deficiency in intraordinal phylogenetic hypotheses. In summary, a well-supported phylogeny of treeshrews, while critical for understanding evolutionary relationships and taxonomy, is also necessary to address character evolution, paleobiology, and biogeography.

### 1.1. Systematic background—higher-level

Monophyly of Scandentia has never been seriously questioned and is supported by several morphological synapomorphies, including: “(1) the enclosure of the intratympanic portion of the internal carotid artery in a bony canal that is floored proximally and distally by the entotympanic and by the petrosal in between, (2) the enclosure of the intratympanic portion of the stapedial artery by the petrosal in a canal on the promontorium and within the epitympanic crest beneath the tympanic roof, (3) the absence of a foramen of exit for the arteria diploëtica magna, (4) an alisphenoid canal, (5) a maxillary artery that passes medial to the mandibular nerve beneath foramen ovale, and (6) a laryngeopharyngeal artery” (Wible and Zeller, 1994, p. 228); (7) scaphoid and lunate fused into scapholunate (Sargis, 2002b); and (8) articular facet for medial malleolus on the posterior side of the sustentaculum (Sargis, 2002a; Szalay and Lucas, 1996). Although some of these character states are also found in Euprimates (e.g., nos. 2, 3, and 6; Wible and Zeller, 1994), others appear to be unique to Scandentia. For example, both the first (#1; Wible, pers. comm.) and last (#8; Szalay, pers. comm.) conditions may represent scandentian synapomorphies that unite *Ptilocercus* and other treeshrews.

While the monophyly of Scandentia is well-supported, the supraordinal relationships of this group have been a matter of great debate (see review in Sargis, 2002a). For example, treeshrews have been proposed to be closely related to euprimates (e.g., Kay et al., 1992; Wible and Covert, 1987), colugos (order Dermoptera; e.g., Liu and Miyamoto, 1999; Liu et al., 2001; Madsen et al., 2001; Murphy et al., 2001a,b; Sargis, 2002a, 2004, in press; Van Den Bussche and Hooper, 2004), and lagomorphs (e.g., Arnason et al., 2002; Graur et al., 1996; Lin et al., 2002; Schmitz et al., 2000). However, the Scandentia + Euprimates clade has not been supported by molecular or morphological evidence (see Bloch and Boyer, 2002; Martin, 1990; Sargis, 2002a, 2004, in press; Silcox, 2001; Silcox et al., in press). Furthermore, some of the analyses that supported a Scandentia + Lagomorpha clade do not represent adequate tests because they either never tested a Scandentia + Dermoptera relationship (e.g., Graur et al., 1996) or they did not include Dermoptera in their analysis (e.g., Lin et al., 2002; Schmitz et al., 2000). However, the Arnason et al. (2002) analysis of 12 mitochondrial protein-coding genes did include Dermoptera and a Scandentia + Lagomorpha clade was still supported. This conflicts with the results of Murphy et al. (2001a), who supported Scandentia + Dermoptera (which we will refer to here as Sundatheria) and Lagomorpha + Rodentia (Glires) clades in their analysis of 19 nuclear and 3 mitochondrial genes, which is the largest molecular dataset to date for placental mammals. Sundatheria, which refers to the distribution of treeshrews and colugos in the greater Sundaland region of Southeast Asia, has been supported by many additional studies (e.g., Liu and Miyamoto, 1999; Liu et al., 2001; Madsen et al., 2001; Murphy et al., 2001b; Sargis, 2002a, 2004, in press; Van Den Bussche and Hooper, 2004), making Dermoptera the most appropriate outgroup for analysis of scandentian intraordinal relationships. In addition to Sundatheria and Glires, Murphy et al. (2001a) also supported Sundatheria + Primates (Euarchonta; see also Liu and Miyamoto, 1999; Liu et al., 2001; Murphy et al., 2001b; Waddell et al., 1999) and Euarchonta + Glires (Euarchontoglires; see also Madsen et al., 2001; Murphy et al., 2001b; Waddell et al., 1999) clades. Despite all these studies of placentalian supraordinal relationships, the *intraordinal* relationships of treeshrews have been largely ignored (see Olson et al., 2004a).

### 1.2. Systematic background—*intraordinal*

The order Scandentia has traditionally included the single family Tupaiidae, which was divided into the subfamilies Tupaiinae and Ptilocercinae by Lyon (1913). This higher-level classification has largely been adopted by subsequent authors (e.g., Corbet and Hill, 1992; Martin, 1990, 2001; Napier and Napier, 1967; Nowak, 1999; Wilson, 1993; see Table 1 for current classification and

Table 1  
Taxonomy of treeshrews

Taxonomy	Accession No.	Geographic distribution
Order Scandentia Wagner, 1855		
Family Tupaiidae Gray, 1825		
<i>Anathana</i> Lyon, 1913		
<i>A. ellioti</i> (Waterhouse, 1850)	AY862169*	India, south of the Ganges River
<i>Dendrogale</i> Gray, 1848		
<i>D. melanura</i> (Thomas, 1892)	AY862167*	Northern Borneo (Malaysia)
<i>D. murina</i> (Schlegel and Müller, 1843)	AY862168*	Southeastern Thailand, Cambodia, Laos, and Vietnam <sup>a</sup>
<i>Tupaia</i> Raffles, 1821		
<i>T. belangeri</i> (Wagner, 1841)	AF217811(1), AY862170(2)*, AY862171(3)*, AY862172(4)*	North of ca. 7°N in Thailand to eastern India, Bhutan <sup>b</sup> , S. China, Myanmar, Laos, Vietnam, and Cambodia, including many adjacent islands
<i>T. chrysogaster</i> Miller, 1903	—	North and South Pagai Islands and Sipora (Mentawai Islands, Indonesia)
<i>T. dorsalis</i> Schlegel, 1857	AY862173*	Borneo (Malaysia, Indonesia, and Brunei)
<i>T. glis</i> (Diard, 1820)	AF153003(1), AY862174(2)*, AY862175(3)*	Southeast Asia south of ca. 10°N in Thailand, through mainland Malaysia to Singapore and several associated islands; Sumatra, Java, and several associated islands (Indonesia)
<i>T. gracilis</i> Thomas, 1893a	AY862176*	Borneo and several associated islands (Malaysia, Indonesia), including Bangka and Belitung
<i>T. javanica</i> Horsfield, 1822	AY862177*	Sumatra, Java, Bali, and Nias (Indonesia)
<i>T. longipes</i> (Thomas, 1893b)	AY862178*	Borneo (Malaysia, Indonesia, and Brunei)
<i>T. minor</i> Günther, 1876	AY012129	Southern peninsular Thailand, Malaysia (Peninsula, Borneo); and Indonesia (Sumatra, Borneo, and several associated islands)
<i>T. moellendorffi</i> Matschie, 1898	—	Calamian Islands and Cuyo (Philippines)
<i>T. montana</i> Thomas, 1892	—	Northern Borneo (Malaysia)
<i>T. nicobarica</i> (Zelebor, 1869)	AY862179*	Great and Little Nicobar Islands (India)
<i>T. palawanensis</i> Thomas, 1894	AY862180*	Palawan and Balabac Islands (Philippines)
<i>T. picta</i> Thomas, 1892	—	Borneo (Malaysia, Indonesia, and Brunei)
<i>T. splendidula</i> Gray, 1865	AY862181*	Southern Borneo and several adjacent islands (Indonesia)
<i>T. tana</i> Raffles, 1821	AF038021(1), AY862183(2)*, AY862182(3)*	Borneo (Malaysia, Indonesia, and Brunei), Sumatra (Indonesia), and several associated islands
<i>Urogale</i> Mearns, 1905		
<i>U. everetti</i> (Thomas, 1892)	AY862184*	Mindanao, Dinagat, and Siargao Islands (Philippines)
Family Ptilocercidae Lyon, 1913		
<i>Ptilocercus</i> Gray, 1848		
<i>P. lowii</i> Gray, 1848	AY862166*	Southern peninsular Thailand, Malaysia (Peninsula, Borneo), Singapore, Brunei, and Indonesia (Borneo, Sumatra, and several associated islands)

Current taxonomy and distribution of treeshrews, following Helgen (in press), and GenBank accession numbers for species included in phylogenetic analyses (asterisks denote new sequences generated in this study; see Appendix A for voucher information). Numbers in parentheses following accession numbers are used in Figs. 1 and 2 to identify individuals of the same species.

<sup>a</sup> From Timmins et al. (2003).

<sup>b</sup> D. Willard and T. Gnoske, pers. comm.

geographic distribution). Recently, Helgen (in press) elevated these two groups to families, Tupaiidae and Ptilocercidae (Table 1). This division, whether at the familial or subfamilial level, has gone largely unchallenged. However, Davis (1938) proposed that Lyon's (1913) division of the family into two subfamilies was invalid because *Dendrogale* is morphologically intermediate between *Ptilocercus* and other treeshrews. This proposal has, however, been repeatedly rejected in other studies of treeshrew morphology and taxonomy (Butler, 1980; Corbet and Hill, 1992; Le Gros Clark, 1926; Luckett, 1980; Martin, 1990, 2001; Napier and Napier, 1967; Nowak, 1999; Steele, 1973; Wilson, 1993). Davis (1938) also suggested that *Dendrogale* may be the most primitive treeshrew, which contradicted

Le Gros Clark's (1926) contention that *Ptilocercus* is the most primitive living treeshrew. Again, Davis' suggestion has not been supported by subsequent studies, which have instead upheld Le Gros Clark's hypothesis (e.g., Emmons, 2000; Martin, 1990; Sargis, 2002a,b, 2004, in press; Szalay and Drawhorn, 1980). The only support for Davis' (1938) hypothesis comes from Stafford and Thorington's (1998) study of carpal morphology in which they proposed that *Dendrogale's* carpus is primitive among treeshrews (but see Sargis, 2002b).

Compared to the few debates regarding the basal relationships among treeshrews, those concerning generic distinction have been more controversial (see Olson et al., 2004a for review). For example, the classification of the

Indian treeshrew *Anathana* as generically distinct from *Tupaia* was questioned by Fiedler (1956). More recently, Helgen (in press) recognized *Anathana* as a separate genus from *Tupaia*, but considered the two taxa to be closely related. However, the relationships of *Anathana* to various species of *Tupaia* have never been explored (see Olson et al., 2004a).

The monophyly of the relatively speciose genus *Tupaia* (including *Lyonogale*; see below) has been challenged by Han et al. (2000), who claimed that *Urogale* should be included in *Tupaia*. *Urogale* was considered to be the sister taxon to a monophyletic *Tupaia* in the immunodiffusion (distance-based) study conducted by Dene et al. (1978, 1980). However, Han et al. (2000) recently proposed, based on DNA hybridization and morphometric analysis of external and cranial characters, that *Urogale everetti* may be nested within *Tupaia*. This conclusion is also supported by postcranial evidence (Sargis, 2004), but all of these contradictory conclusions must be considered with caution because they are the results of phenetic analyses with extremely limited taxon sampling. Only a phylogenetic analysis that includes data from several more taxa will resolve the issue of the monophyly of *Tupaia*.

In addition to questions regarding the monophyly of *Tupaia*, the number of species included in this genus has varied from 32 (Lyon, 1913) to 11 (e.g., Corbet and Hill, 1992; Martin, 1990, 2001; Nowak, 1999) in the last 90 years. Within Scandentia, 120 described forms (species or subspecies; not including fossils) have been proposed since the first species of treeshrew was described in 1820. A full review of this tumultuous taxonomic history is beyond the scope of this paper, and we agree emphatically with Wilson (1993) that a thorough taxonomic review is desperately needed.

One final problem in treeshrew systematics, beyond taxonomic recognition of various genera and species, is that phylogenetic analyses based on morphological data have not resolved intergeneric relationships (Olson et al., 2004a). Steele's (1973) cluster analysis was problematic because *Anathana* was not included in his study. Butler (1980) and Lockett (1980) both included *Anathana* in their analyses, but they also assumed generic monophyly (including the questionable genus *Lyonogale*; see below). Furthermore, their cladistic analyses produced different results regarding the relationships of treeshrew genera. Our reanalysis of the data from these three studies showed that the most parsimonious trees were not published in the original papers (Olson et al., 2004a), but even our parsimony analyses failed to resolve intergeneric relationships. In addition to these problems, the original studies did not include non-scandentian outgroups, nor did they agree on character codings (Olson et al., 2004a). We have begun to address these problems in a forthcoming study on the morphological systematics of treeshrews (Sargis and Olson, in prep.) that will

include outgroups, identify voucher specimens, investigate character state variation, and test (rather than assume) generic monophyly.

### 1.3. Current objectives

To address several of these issues, we have conducted a phylogenetic analysis of all living treeshrew genera and all but four species based on variation in the mitochondrial 12S rRNA gene (12S hereafter). Several characteristics of 12S make it a desirable marker for investigating phylogenetic relationships among mammals. First, it is extremely well-characterized in mammals due to its popular use for over a decade in studies of mammalian interrelationships (e.g., Miyamoto et al., 1989; Rowe and Honeycutt, 2002; Springer and Kirsch, 1993). The secondary structure of the small ribosomal subunit in mammals (encoded by the 12S gene) has been rigorously modeled by Springer and Douzery (1996), allowing alignments based on structural features rather than simple sequence similarity alone, which has been shown to improve phylogenetic inference (Kjer, 1995, 2004). Second, 12S is characterized by regularly occurring conserved regions (Hickson et al., 1996; Springer et al., 1995; Springer and Douzery, 1996), making it possible to design PCR primers that will amplify overlapping fragments of varying length. Because no fresh tissues are available for a number of treeshrew species whose inclusion in this study is critical to our objectives, the ability to amplify short pieces of mtDNA is a distinct advantage given the availability of degraded tissues often present on museum specimens. Finally, while relatively conserved regions of the gene tend to be useful for recovering deeper nodes in phylogenetic analysis, more variable positions, usually found in non-pairing regions of the transcribed molecule (particularly loops; e.g., Springer et al., 1995), extend the utility of 12S to more recently divergent nodes as well. We have taken advantage of these features of the 12S gene to infer the phylogenetic relationships among treeshrews. Our specific objectives are to: (1) explore the widely hypothesized but heretofore untested phylogenetic position of *Ptilocercus* as the sister taxon to all other living treeshrews; (2) determine whether *Dendrogale* is more closely related to other tupaiids or to *Ptilocercus*; (3) elucidate relationships among species in the taxonomically diverse genus *Tupaia*; and (4) determine whether the resulting phylogeny places the controversial genera *Anathana* and *Urogale* within the genus *Tupaia*, as some authors have suggested (see above).

## 2. Materials and methods

### 2.1. Taxon sampling, DNA amplification, and sequencing

Complete sequences from the mitochondrial 12S rRNA gene were obtained from GenBank, museum

specimens, and zoo animals (see Table 1 and Appendix A) representing 23 individuals assigned to 16 species. Of the 20 currently recognized species of treeshrew, 4 are not represented in this study due to our unsuccessful attempts to obtain material suitable for DNA extraction (see Table 1). DNA from 10 of the museum specimens and the single zoo animal was extracted from fresh frozen muscle or organ tissues using the animal tissue protocols in the PureGene (Gentra Systems, Inc.) or Qiagen DNeasy (Qiagen) kits.

For the remaining six taxa for which fresh tissues were unavailable, dried tissue (ca. 6–20 cm<sup>3</sup>) picked from museum skulls and/or skeletons was used for DNA extractions. Samples were soaked overnight in sterile water at room temperature, decanted, washed repeatedly in 70% ethanol (with vigorous agitation), and air-dried at room temperature for several hours. The PureGene kit was then used to extract DNA following the animal tissue protocol, with additional proteinase-K added at half day intervals as necessary until all tissue had been digested (up to several days). Purified DNA was re-suspended in 30 µL water, but the final concentration used varied widely, with some samples yielding PCR products only when diluted 10- or 100-fold.

Amplifications were performed following Olson et al. (2004b) for fresh tissue extractions. Cycling parameters (including annealing temperature), MgCl<sub>2</sub> concentration (1.5–3 mM), and template volumes were adjusted as necessary for dried tissue extractions and often differed depending on the primer combinations employed. Ampli-Taq Gold polymerase (Perkin-Elmer) was used in varying concentrations for the degraded samples; custom-made *Taq* was used for all fresh samples.

Different PCR and sequencing strategies were employed according to the quality of the extracted DNA. For relatively intact, high-quality DNA (e.g., from fresh tissues), the entire 12S gene was amplified using primers located on the tRNA-Phe gene (5' of 12S) and the tRNA-Val or 16S gene (3' of 12S). Either two or three separate sequencing reactions were performed on each purified PCR product using the same flanking primers as those used in the initial PCR, with two or four overlapping internal primers, respectively. For degraded DNA, much shorter pieces had to be amplified (ranging from 75 to 389 bases, not including primer sequences). In all, 33 different primers were used in various combinations in this study, many of which were designed specifically for treeshrews based on sequences from GenBank and fresh samples. Because of the degraded nature of the DNA extracted from dried tissues, as many as 10 individual, overlapping PCR products were necessary to reconstruct the entire 12S gene for some specimens. Primer sequences and their location on the published mitochondrial genome of *Tupaia belangeri* (GenBank Accession AF217811) are given in Appendix B. A schematic representation of the primer combina-

tions used to generate sequences from degraded samples is provided in Appendix C.

All PCR products were purified using the GeneClean protocol (Bio101) following the manufacturer's instructions. Unquantified aliquots of purified PCR product (1–5 µL) were cycle-sequenced for both strands using the BigDye Ready Reaction Kit (Applied Biosystems) following the manufacturer's directions, other than using 10 µL reaction volumes with reagents scaled down accordingly. Sequencing reactions were purified using either ethanol or ethanol/NaAc precipitation. Samples were electrophoresed on either an ABI 377 or 3100 automated sequencer (Applied Biosciences, Perkin-Elmer). The resulting sequences were edited and compiled using Sequencher 4.1 (GeneCodes). All sequences in this study have been deposited to GenBank (accession numbers are given in Table 1).

## 2.2. Sequence verification

Concatenating multiple, short sequence fragments generated from degraded DNA into longer continuous sequences is fraught with potential problems (Hassanin, 2002; Olson and Hassanin, 2003). Foremost among these is the risk of contamination from a number of sources (e.g., human DNA, ambient PCR products, and purified DNA from other extractions) and the subsequent inadvertent reconstruction of chimaeric sequences (Hassanin, 2002; Olson and Hassanin, 2003). While a number of studies and commentaries have emphasized the issue of contamination and have suggested rigorous measures for preventing it (e.g., Cooper and Poinar, 2000), relatively few papers have addressed the equally critical need for verifying sequence authenticity and preventing the unintentional concatenation of chimaeric sequences (Hassanin, 2002; Olson and Hassanin, 2003). Following standard procedures for safeguarding against contamination from extraneous DNA can be considered a separate issue from the risk of amplifying non-target DNA present in a given sample. This is particularly true when DNA is extracted from archival museum specimens, which may have been handled by several individuals during the course of preparation and subsequent examination, in addition to having been subjected to either microbial digestion (maceration) or dermestid beetle skeletonization. Thus, even when negative controls (extraction and amplification) performed in parallel with the same steps conducted on a sample obtained from a museum skull or skeleton yield no PCR product(s), the sample itself may still be contaminated with human, bacterial, and/or invertebrate DNA. Amplifying short fragments of genes characterized by multiple highly conserved regions, such as 12S, compounds this risk. For example, the popular primers published in Kocher et al. (1989) spanning the conserved third domain of the 12S rRNA gene will readily amplify 12S from a wide range of

vertebrate taxa (Kocher et al., 1989), as well as the corresponding region in the homologous 16S rRNA gene in some bacteria (Olson, unpubl.). Finally, the amplification of mitochondrial-derived nuclear pseudogenes (numts) of 12S from degraded samples was reported by Van der Kuyl et al. (1995). The identification of numts in ribosomal genes can be exceedingly difficult (Olson and Yoder, 2002), making this a particularly problematic issue. Taken together, these concerns warrant very careful examination of resulting sequences, particularly those derived from short amplification products. Towards that end, we propose (and have followed) the following steps for determining whether such sequences are authentic. We emphasize, however, that none of these measures, individually or collectively, is failsafe.

In addition to performing standard negative controls during both the extraction and amplification processes (wherein separate PCRs were performed using the negative control extraction and sterile water in lieu of extracted DNA), we critically examined each individual sequenced PCR product (i.e., prior to concatenation) using the following protocol: first, each sequenced fragment was subjected to an unconstrained BLAST search (Altschul et al., 1997). The 12S rRNA gene is perhaps the most widely sequenced gene (taxonomically) in mammals, with every currently recognized order and most families represented on GenBank. The same is true, to a lesser extent, for several other vertebrate and invertebrate taxa. As such, a simple BLAST search is a logical first step towards checking for contamination. Second, each fragment was compared to all other 12S sequences generated by L.E.O. (not restricted to those produced for this study alone) using Sequencher. Comparisons were made not only to all individual sequenced PCR products, but to all concatenated sequences as well, since different primer pairs were used for different specimens. Finally, the structural model for 12S rRNA proposed by Springer and Douzery (1996) was used to check for positional covariation between sites residing on different PCR products normally engaged in canonical (and in some cases non-canonical) pairing, although this by no means guarantees detection of contamination by extraneous DNA or pseudogenes (see Olson and Yoder, 2002).

### 2.3. Data analysis

Sequences were aligned by eye with reference to the secondary structure model of Springer and Douzery (1996). Regions that could not be confidently aligned (primarily indel-rich regions in loops) were excluded from all analyses, as were partial tRNA sequences at either end of the 12S rRNA gene, since the length of these flanking sequences varied depending on the amplification primers used. Base composition of all taxa was examined to test for significant heterogeneity, which has been shown to affect phylogenetic inference (e.g., Lock-

hart et al., 1994). We assessed compositional homogeneity across the entire 12S gene as well as within stem and loop regions separately for all individual taxa using a  $\chi^2$  goodness of fit test. Phylogenetic analyses were performed under both maximum parsimony (MP) and maximum likelihood (ML) using the software PAUP\* 4.0b10 (Swofford, 2003).

For MP analyses, all characters were treated as unordered and equally weighted. Tree searches were conducted using the branch-and-bound algorithm, an exact method guaranteed to find all optimal trees. Nodal support was estimated by heuristic bootstrap re-sampling of 1000 pseudoreplicates employing the TBR branch swapping algorithm. Because transitions have been shown to become saturated in mammalian 12S sequences after 20 million years of divergence within loop and, to a lesser extent, stem regions (Springer and Douzery, 1996), and because fossil evidence demonstrates that treeshrews originated well before 20 million years ago (see reviews in Sargis, 1999, 2004), we conducted separate tree-search (branch-and-bound) and bootstrap (TBR) analyses of transversional changes only (TvMP hereafter). Bremer support values (Bremer, 1994) were calculated for select groupings using the “constraints” option in PAUP\* under otherwise identical search conditions. Uncorrected pairwise distances were calculated for select inter-taxon comparisons.

For ML tree searches, the best-fit model of nucleotide substitution was estimated using the successive approximation approach suggested by Swofford et al. (1996). A single MP tree was used to estimate likelihood model parameters under a nested series of substitution modes using the program Modeltest 3.0 (Posada and Crandall, 1998, 2001). Parameter values based on the preferred model according to the likelihood ratio test were then fixed in a ML search employing 20 heuristic replicates with TBR branch-swapping in PAUP\*. The resulting tree was used to re-estimate model parameters, which were fixed for a second round of tree searches. This process was repeated until a stable topology was obtained. Bootstrap support was evaluated under the final substitution model for 1000 pseudoreplicates using the TBR branch-swapping algorithm on starting trees obtained by stepwise addition.

In addition to bootstrap proportions, Bayesian posterior probabilities were estimated to assess nodal support using MrBayes (v. 3.0; Ronquist and Huelsenbeck, 2003). Mutations at covarying positions (e.g., stem sites involved in canonical pairing in the transcribed rRNA molecule) violate the fundamental assumption of character independence in phylogenetic analysis (Dixon and Hillis, 1993). While some authors have recommended down-weighting these sites to adjust for their evolutionary non-independence (e.g., Dixon and Hillis, 1993), others have argued that such positions tend to be conserved and, as such, should be accorded greater weight due to

the lower likelihood of parallelisms and reversals (e.g., Fitch, 1992; Miyamoto et al., 1994). Springer et al. (1995) advocated a more integrative (but unspecified) approach, showing that simple weighting factors are insufficient to accommodate the evolutionary complexities of molecules such as 12S. We took advantage of the recently implemented feature in MrBayes that allows for the explicit incorporation of a secondary structural model. To assess the effect of adding structural interactions to the model, we conducted separate Bayesian analyses with and without specification of the secondary structure model of Springer and Douzery (1996). For both analyses, four MCMC chains (three heated, one unheated) were allowed to proceed for 10 million generations, sampling trees every 100 generations. In the first analysis, a model with six categories of base substitution was specified, with a gamma-distributed rate parameter and a proportion of invariant sites. In the second analysis, two data partitions were recognized (pairing and non-pairing) and model parameters for each partition were estimated separately (i.e., “unlinked”). The same model as in the first analysis was applied to the non-pairing partition. For the pairing partition, the doublet model of nucleotide substitution, which is appropriate for modeling stem regions of ribosomal genes, was specified. Trees sampled prior to the attainment of stable likelihood values were discarded as burn-in and all subsequently sampled trees in each MCMC run were included in 50% majority-rule consensus trees to obtain posterior probabilities.

Separate analyses were conducted using either primates (*Homo sapiens*, GenBank Accession V0062; *Otolemur crassicaudatus* AF179289), lagomorphs (*Oryctolagus cuniculus* AJ001588; *Ochotona princeps* AF390540), or dermopterans (colugo; *Cynocephalus variegatus* AF038018) to root trees in order to evaluate the effects of different outgroups on tree topology.

### 3. Results

#### 3.1. Sequence verification

None of the negative extraction or PCR controls yielded amplification products for any combination of primers used. When individual sequenced PCR products from the degraded samples were subjected to BLAST searches (Altschul et al., 1997), all of the sequences longer than 100 nucleotides for *Anathana*, *Tupaia javanica*, and *T. nicobarica* were most similar (but not identical) to one of the four *Tupaia* 12S sequences on GenBank (see Table 1). The same was true for all fragments >200 bases sequenced for both species of *Dendrogale*. Most shorter fragments (and all those sequenced for *Ptilocercus*) were between 85 and 98% similar to non-scandentian mammals, but this is not surprising given

the length of some of these sequences (as short as 75 bases), the numerous conserved regions in the 12S gene, and the expected levels of divergence between *Ptilocercus*, *Dendrogale*, and published *Tupaia* sequences. Most notably, none of the sequences subjected to BLAST searches were 100% similar to any GenBank sequence. The same was true for the comparisons between individual sequenced PCR products and all other sequences generated for this study. Every fragment sequenced in this study was unique. Finally, reference to secondary structure did not suggest chimaeric concatenation of individual fragments within a single reconstructed (complete) 12S sequence. While these measures, either individually or collectively, are by no means definitive or failsafe, we nonetheless conclude that we have no evidence of contamination from either extraneous DNA or pseudogenes in the sequences generated for this study. Details on the amplification and sequencing strategy employed for all specimens sequenced in this study are provided in Appendix C.

#### 3.2. Sequence characteristics

The length of the 12S gene in treeshrews ranged from 944 to 960 nucleotides. The alignment to secondary structure with all outgroups included was 1004 bases (Appendix D). Because individual analyses using either primates or lagomorphs as outgroups (results not shown) did not result in different ingroup topologies from those using colugo as the outgroup, only the results of the analyses with colugo as the outgroup will be discussed hereafter. Nucleotide frequencies in treeshrew 12S sequences are very similar to those reported by Springer et al. (1995) for mammalian 12S, with adenine as the most abundant nucleotide (mean = 33.4%), followed by cytosine (23.7%), thymine (22.6%), and guanine (20.3%). A similar preponderance of adenine in loop regions (mean = 44.7%) was also found.

The exclusion of alignment-ambiguous positions resulted in 827 included characters comprising 256 variable positions (96 transversions), 166 (55 transversions) of which were parsimony informative. Uncorrected pairwise distances including all changes ranged from 14.6 to 17.5% between *Cynocephalus* and all treeshrews; 14.4 to 16.2% between *Ptilocercus* and all tupaiids; 6.7 to 12.6% between both species of *Dendrogale* and the remaining tupaiids; and 2.3–7.7% among all species of *Tupaia* as well as *Anathana* and *Urogale*. Intraspecific divergences ranged between 0.2 and 1.5% in the three species for which sequences were available from multiple individuals (see Table 1).

#### 3.3. Phylogenetics

The branch-and-bound search including all changes under the parsimony criterion yielded 66 equally parsim-

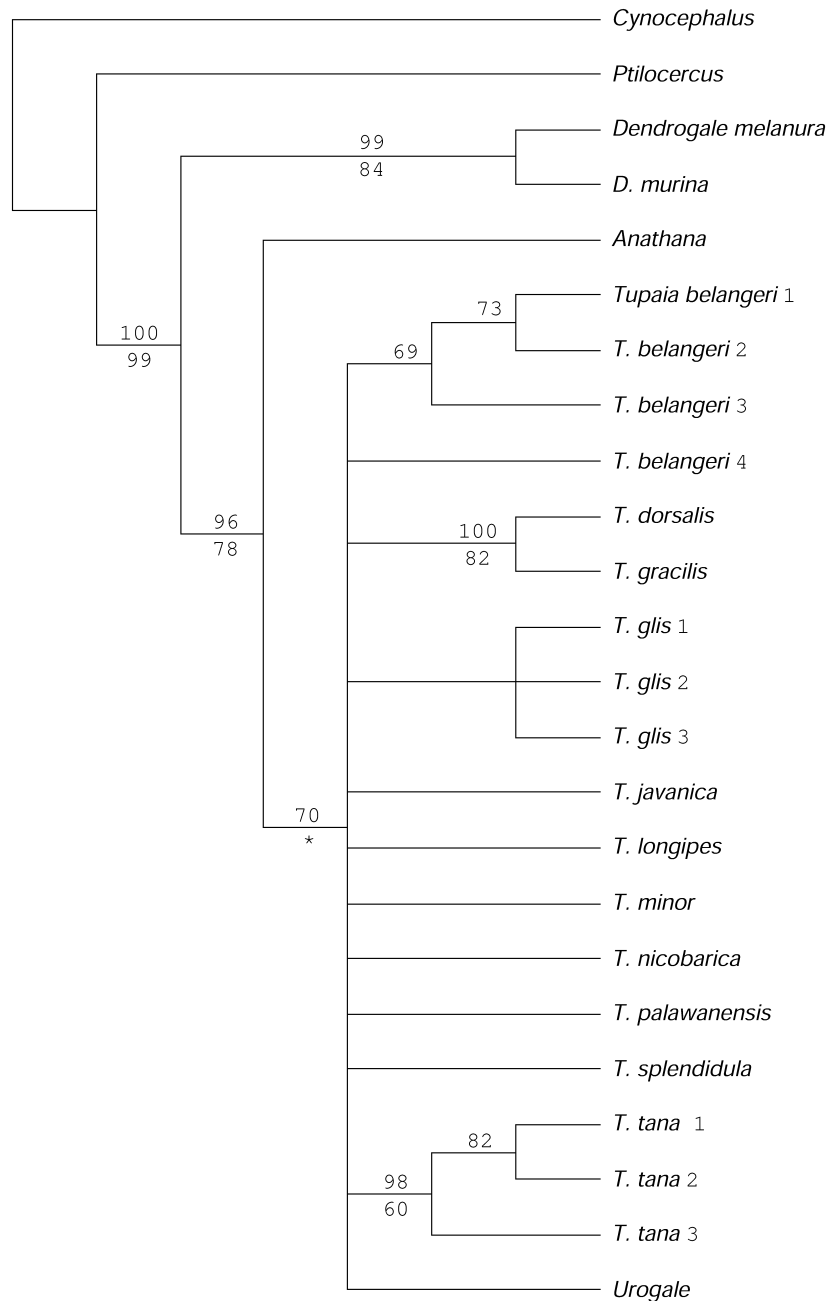


Fig. 1. Strict consensus tree representing the 66 most parsimonious trees recovered in the MP branch-and-bound tree search. Numbers above branches represent bootstrap proportions from the MP bootstrap analysis; those below branches represent bootstrap support in the TvMP analysis. The node indicated by an asterisk was not recovered in the latter. Numbers to the right of some species names are used to identify specimens of the same species (see Table 1). Specific epithets are not shown for monotypic treeshrew genera.

monious trees of 621 steps. A strict consensus of these with bootstrap support values is shown in Fig. 1. The TvMP search resulted in only four equally parsimonious trees (157 steps; not shown). None of the TvMP trees was identical to any of the minimal length trees obtained in the MP search, but the same three basal splits were recovered in both analyses (Fig. 1). A monophyletic Tupaiidae (sensu Helgen, in press; Olson et al., 2004a) was recovered in 100% (MP) and 99% (TvMP) of the bootstrap replicates, with either slightly (96%; MP) or

notably (78%; TvMP) lower support for the basal divergence of *Dendrogale* relative to the remaining tupaiids. Although all MP trees favored *Anathana* as the sister taxon to a clade containing all *Tupaia* specimens as well as *Urogale*, MP bootstrap support for the latter clade was marginal (70%). In contrast, *Anathana* was recovered as nested within either *Tupaia* (two trees, with *Urogale* as the next basal split) or *Tupaia* + *Urogale* (two trees) in the TvMP analyses, although bootstrap support was low for all interspecific groupings in the

*Tupaia* + *Anathana* + *Urogale* clade when transversions only were counted. The only noteworthy difference between the two parsimony analyses with respect to this clade was the sister relationship between *T. splendidula* and the three specimens of *T. tana*, which was recovered in 70% of the TvMP bootstrap replicates but in fewer than 50% of the MP replicates. No other nodes with >70% bootstrap support (an arbitrary cutoff) were recovered in the TvMP analyses that were not recovered with equal or greater support in the MP analyses. However, one clade received 60% bootstrap support in TvMP but was not recovered in any of the optimal MP trees (and is thus not labeled in Fig. 1); this grouping is discussed below with results from the Bayesian analysis.

Likelihood analyses converged on a stable topology after a single iteration of the model estimation procedure. The preferred model of nucleotide substitution was equivalent to the TrN (Tamura and Nei, 1993) model with site-specific rate variation approximated by the gamma ( $\Gamma$ ) distribution (gamma distribution shape parameter = 0.397) with a proportion of invariant sites ( $I = 0.479$ ) and no molecular clock. The single ML tree ( $-\ln L = 3925.66$ ) with associated ML bootstrap values is shown in Fig. 2. The same basal relationships (*Ptilocercus* (*Dendrogale* (*Anathana* (*Tupaia* + *Urogale*)))) were recovered as in the MP analysis, with similarly high bootstrap support (98%) for a monophyletic Tupaiidae. Unlike both MP and TvMP, however, a basal divergence of *Dendrogale* relative to the remaining tupaiids was not recovered in a majority of the bootstrap replicates. Likewise, *Anathana* was favored as the sister taxon to *Tupaia* + *Urogale* but did not receive high bootstrap support.

Likelihood scores converged on a stable value before 150,000 generations had elapsed in both Bayesian analyses; the first 200,000 generations were therefore excluded as burn-in. Resulting posterior probabilities are shown on the ML tree in Fig. 2. Support was similar in both Bayesian analyses for well-supported ( $\geq 0.95$  posterior probability) nodes, suggesting that non-independent positions are not spuriously inflating support for these clades in the analysis that did not incorporate secondary structural interactions. Indeed, differences in posterior probability for these nodes did not exceed 0.01. Otherwise, such differences were inconsistent, with approximately half of the remaining nodes receiving higher support in one analysis or the other (though we reiterate that none of these was well-supported in either analysis, making such comparisons tenuous). The only two of these that we consider noteworthy are those uniting *Anathana* + *Tupaia* + *Urogale* and the more nested grouping of *T. minor* + *T. splendidula* + *T. tana*. Both have higher posterior probabilities in the analysis without secondary structure (0.85 and 0.89 versus 0.65 and 0.67, respectively). This is to be expected if a significant number of the mutations that accumulated along each

branch leading to these clades occurred in stem regions and were compensated for prior to the subsequent diversification, thereby increasing perceived support through non-independent changes. This may also explain the strong support for the *Anathana* + *Tupaia* + *Urogale* clade in MP (and, to a lesser extent, TvMP; Fig. 1).

Only one clade was supported by a high posterior probability that did not receive bootstrap support  $\geq 70\%$  in MP, TvMP, or ML. This node, indicated by the black square in Fig. 2, unites six species of *Tupaia* with posterior probabilities of 1.0 and 0.99 in the analyses with and without secondary structure, respectively. The single ML tree, 8 of the 66 MP trees, and all optimal TvMP trees recovered this clade, but only in the latter did it receive 60% bootstrap support. While some have suggested that Bayesian posterior probabilities inflate nodal support (e.g., Suzuki et al., 2002), the recent study by Alfaro et al. (2003) found general concordance between support values generated by non-parametric bootstrapping (under both parsimony and likelihood) and posterior probabilities. They did, however, identify several scenarios in which strong ( $\geq 0.95$ ) Bayesian support was found for nodes not supported by high bootstrap values. These include regions of a tree with short internodes and a relatively small number of characters. This may well be the case for the node in question. The majority of the branches in the *Tupaia* + *Urogale* clade are relatively short in the ML tree (Fig. 2), and of the 166 parsimony informative characters in the complete taxon sample, fewer than half (86) are informative in this group. We therefore provisionally accept this explanation for the disparity in support estimates, as well as the clade itself, though additional data will likely be required to rigorously confirm it (Alfaro et al., 2003).

## 4. Discussion

### 4.1. Tupaiid monophyly

Monophyly of Tupaiidae (sensu Helgen, in press; Olson et al., 2004a) was recovered in all phylogenetic analyses with strong support, regardless of the outgroup. This is not surprising in light of the numerous morphological differences separating *Ptilocercus* from all other treeshrews (e.g., Lyon, 1913; Sargis, 2000, 2002a,b, 2004). However, the only published studies of intergeneric relationships among treeshrews have either assumed a basal divergence for *Ptilocercus* (e.g., Butler, 1980; Luckett, 1980; Steele, 1973) or have not included it (e.g., Dene et al., 1978; Han et al., 2000). As such, this is the first study to provide phylogenetic support, molecular or otherwise, for the basal divergence of *Ptilocercus* from the lineage leading to all other living treeshrews. While neither topology nor levels of sequence divergence are appropriate criteria for justifying the familial status of

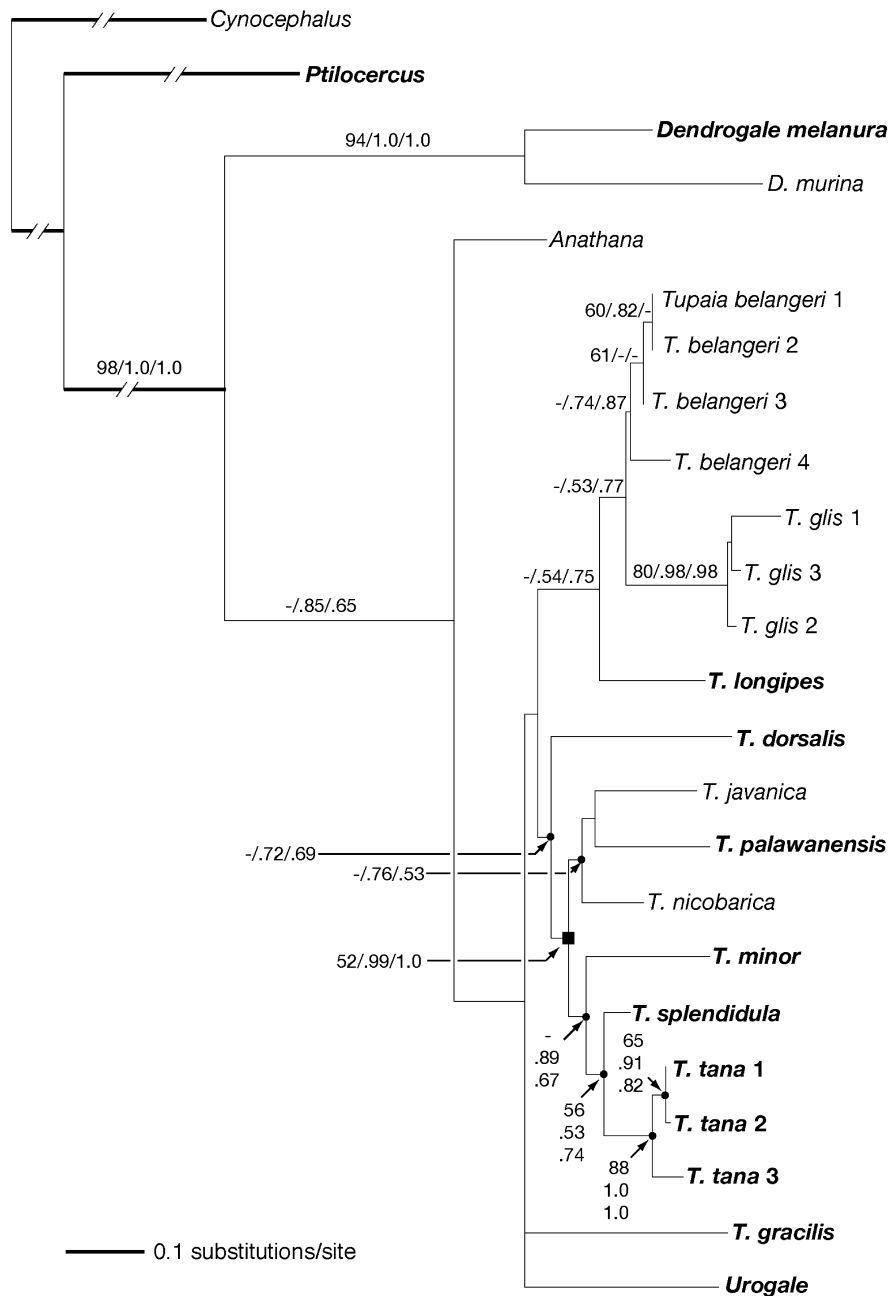


Fig. 2. Single optimal tree recovered in the heuristic ML search. Numbers to the left of nodes represent bootstrap proportions from ML bootstrap analysis followed by Bayesian posterior probabilities without and with the incorporation of the secondary structural model, respectively. Dashes indicate nodes that did not receive >50% bootstrap support or were not resolved in the majority-rule consensus tree from MrBayes. Branch lengths are scaled proportional to the number of substitutions per site, with the exception of the bold branches (additionally identified by back slashes), which are one-quarter actual length. Numbers to the right of species names are as in Fig. 1. Species known to occur on (but not necessarily endemic to) Borneo are shown in bold (*Urogale* and *Tupaia palawanensis* are considered here to be Bornean in origin [see Sargis, 2000]). The node identified by the solid square represents conflicting results in ML and Bayesian analyses and is discussed in the text.

Ptilocercidae, we nonetheless note that the levels of divergence observed between *Ptilocercus* and Tupaiidae for the 12S gene (14.4–16.2% uncorrected pairwise distance) are on par with those between the two families of primates we included as alternative outgroups (16.2%) and in excess of those observed between the two lagomorph families, Leporidae and Ochotonidae (13.5%).

Collectively, the morphological (Sargis, 2000, 2002a,b, 2004) and molecular evidence make a compelling case for recognizing Ptilocercidae as a distinct family.

Davis (1938) rejected Lyon's (1913) division of tree-shrews into two subfamilies (Ptilocercinae and Tupaiinae) based on his interpretation of *Dendrogale* as either morphologically intermediate between *Ptilocercus* and

Tupaiainae (equivalent to Tupaiaidae here) or more primitive morphologically than all other treeshrews including *Ptilocercus* (see above). However, subsequent authors have not endorsed this view (but see Stafford and Thorington, 1998). While MP, ML, and Bayesian analyses of the 12S data reported here all strongly support tupaiaid monophyly, less consistent support was found for a basal divergence of *Dendrogale* with respect to the remaining tupaiaids. Although this relationship was recovered in all MP trees and the optimal ML tree, as well as in 96% of the MP bootstrap pseudoreplicates, it received less than 50% bootstrap support in the ML analyses. Bayesian posterior probabilities were less than 0.95 in both analyses (with and without secondary structural interactions specified in the model). Our previous reanalysis of two published morphological datasets (Butler, 1980; Luckett, 1980) supported *Dendrogale* as the sister taxon to the remaining tupaiaids (Olson et al., 2004a). However, both datasets were rooted with *Ptilocercus* since neither author had included outgroups in their original dataset. (Additional problems with these datasets were discussed by Olson et al. (2004a), including questionable assumptions of generic monophyly and differences in character conceptualization.) Given the strong support here for tupaiaid monophyly, the use of *Ptilocercus* to root the trees in Olson et al. (2004a) can now be considered defensible. As such, we consider the morphological data published in Butler (1980) and Luckett (1980) as supportive of *Dendrogale*'s basal divergence among tupaiaids. Taken together with the similar, if moderately well-supported, relationships recovered here, we believe the evidence adduced to date for *Dendrogale*'s phylogenetic position depicted in Figs. 1 and 2 outweighs any evidence to the contrary. We therefore reject Davis' (1938) interpretation of *Dendrogale* as the basal scandentian, as all known evidence supports the proposal that *Ptilocercus* occupies this position (e.g., Le Gros Clark, 1926; Emmons, 2000; Martin, 1990; Sargis, 2000, 2002a,b, 2004, in press; Szalay and Drawhorn, 1980).

#### 4.2. The enigmatic Indian treeshrew

The generic status of the Indian treeshrew, *Anathana*, was called into question by Fiedler (1956), who considered it a potential congener of *Tupaia*. Similarly, Helgen (in press) suggested a close relationship between these genera. Our analyses recovered *Anathana* as the sister taxon to the clade containing *Tupaia* and *Urogale*, although neither bootstrap re-sampling (MP and ML) nor Bayesian analysis provides strong support for this. In the absence of any conflicting evidence, we tentatively accept the arrangement shown in Figs. 1 and 2 with respect to *Anathana*. The only other treeshrew species known to occur on mainland southeast Asia north of the Isthmus of Kra are *Tupaia belangeri* and *Dendrogale*

*murina*, with the latter known only from southeastern Thailand, Cambodia, Laos, and Vietnam (Timmins et al., 2003). Based solely on current geographic distributions, it might be expected that *Anathana* is closely related to *T. belangeri*, which is known to occur in eastern India (Helgen, in press; Lyon, 1913; Martin, 2001; Roonwal and Mohnot, 1977) and Bhutan (D. Willard and T. Gnoske, pers. comm.). However, a number of characters differentiate *Anathana* from all species in the genus *Tupaia* (Lyon, 1913). Low support values notwithstanding, our results do not support a close relationship between *Anathana* and any extant treeshrew, although MP analyses constrained accordingly recover trees in which *Anathana* is not basal to *Tupaia*+*Urogale* at a cost of only two additional steps, with *Anathana* falling out as either the sister taxon to *Urogale* or nested within the *Urogale*+*Tupaia* clade. The circumstances resulting in *Anathana*'s limited and comparatively isolated distribution (India, south of the Ganges) remain a mystery for which our results offer no clear explanation. It is, however, worth noting that fossil treeshrews have been recovered from Miocene deposits of the Siwaliks in India and Pakistan (Chopra and Vasishat, 1979; Chopra et al., 1979; Jacobs, 1980; Sargis, 1999, 2004).

#### 4.3. Relationships within *Tupaia*

Monophyly of the relatively speciose genus *Tupaia* has been questioned with respect to both *Anathana* (Fiedler, 1956; see above) and *Urogale* (Han et al., 2000; see above). Dene et al. (1978, 1980) reported an immunological distance-based tree suggesting *Urogale* as the sister taxon to a monophyletic *Tupaia*, although their limited taxon sample (neither *Anathana* nor *Dendrogale* were included, and only seven species of *Tupaia* were available) makes such conclusions tenuous. This same issue applies to the study by Han et al. (2000), who concluded that *Urogale* may be nested within *Tupaia* based on morphometry (see also Sargis, 2004; for postcranial evidence) and DNA hybridization. Neither the original cluster (UPGMA) analysis of Steele (1973), nor our (Olson et al., 2004a) recent parsimony analysis of his dataset (comprising 43 discrete dental characters) supported a monophyletic *Tupaia*, with both *Urogale* and *Dendrogale* recovered as nested within *Tupaia* (but see Olson et al., 2004a, for a discussion of character-coding issues). Although some authors have either implicitly or explicitly assumed monophyly of *Tupaia* (e.g., Butler, 1980; Luckett, 1980), there seems to be relatively little supportive data (Olson et al., 2004a).

*Urogale everetti* was originally placed in the genus *Tupaia* by Thomas (1892) in his description of the species. That Thomas, a keen observer of (and prolific publisher on) morphological variation in mammals, chose not to recognize this species as a new genus is noteworthy. Mearns (1905) inexplicably erected the genus

*Urogale* in his description of *U. cylindrura* (subsequently synonymized with *U. everetti*). Lyon's (1913) description of *Urogale*'s morphological distinctiveness remains the most thorough to date, and several characters contrast strikingly with their homologs in other genera, including an elongate rostrum (which may be related to *Urogale*'s large body size and rooting behavior; Martin, 1990, 2001) and enlarged second upper incisor. Lyon (1913) upheld its generic status (as have most subsequent authors; e.g., Butler, 1980; Corbet and Hill, 1992; Dene et al., 1978, 1980; Helgen, in press; Luckett, 1980; Martin, 1990, 2001; Napier and Napier, 1967; Nowak, 1999; Steele, 1973; Wilson, 1993) and regarded *Urogale* as more different from *Tupaia*, *Anathana*, and *Dendrogale* than any of these genera are from each other. However, he also opined that *Urogale* shared a common ancestor with *T. tana* and *T. dorsalis* to the exclusion of all other *Tupaia* species, a hypothesis that has been rejected repeatedly (e.g., Butler, 1980; Dene et al., 1978, 1980; Luckett, 1980). Our results support neither a *T. tana* + *T. dorsalis* clade, nor a sister relationship between *Urogale* and either of these species.

The confusing history of the name *Lyonogale* Conisbee 1953 was recently reviewed by Olson et al. (2004a). In short, Lyon (1913) erected the genus *Tana* to accommodate the species now referred to *Tupaia tana* and *T. dorsalis*. Chasen (1940) reallocated these taxa to the genus *Tupaia*, though Ellerman and Morrison-Scott (1951) retained *Tana* as a subgenus comprising these two species. *Tana* was later renamed *Lyonogale* by Conisbee (1953), noting the preoccupation of *Tana* by a fly. Some subsequent authors have accepted *Lyonogale* as a valid taxon (e.g., Butler, 1980; Luckett, 1980; Martin, 1968, 1990, 2001; Napier and Napier, 1967), while others have rejected it (e.g., Corbet and Hill, 1992; Helgen, in press; Nowak, 1999; Wilson, 1993). Dene et al. (1978, 1980) proposed including *Tupaia montana*, *T. minor*, and *T. palawanensis* with *T. tana* in the subgenus *Lyonogale* based on immunological distances, but *T. dorsalis* was not included in these analyses. Similarly, our taxon sample did not include *T. montana*, so we are unable to comment on this latter hypothesis. But, while we found no strong support for or against a sister relationship between *T. dorsalis* and *T. tana*, this grouping was not favored by ML and was not recovered in any of the shortest MP or TvMP trees. (MP searches constrained to recover a *T. dorsalis* + *T. tana* clade were seven steps longer; trees obtained from a similarly constrained TvMP analysis were 5 steps longer.) Perhaps more importantly, both of these species were nested among species of *Tupaia*, so we reject the recognition of *Lyonogale* as a separate genus.

In addition to the lack of support for *Lyonogale*, all analyses except MP favored *T. splendidula* as the sister taxon to *T. tana*, and this clade was recovered in 16 of the 66 optimal trees in MP analyses (although none of

the analyses provided strong support for this clade). The status of *T. splendidula* as a distinct species was first questioned by Thomas and Hartert (1894), who identified one of the two type specimens as a juvenile *T. tana*, an observation later confirmed by Hill (*in litt.*, as cited in Medway, 1961). Chasen (1940), while recognizing *T. splendidula*, nonetheless suggested that it was more likely conspecific with *T. glis*. Medway's (1961) study, based on additional specimens, confirmed the morphological distinctiveness of *T. splendidula*, and subsequent classifications have recognized this species (e.g., Corbet and Hill, 1992; Helgen, in press; Martin, 1990, 2001; Napier and Napier, 1967; Nowak, 1999; Wilson, 1993). Despite our lack of support for *T. splendidula*'s phylogenetic position, our sample of *T. tana* bears on this issue. Neither locality nor voucher information were provided for the previously published 12S sequence for *T. tana* (GenBank Accession AF038021), but the two specimens we sequenced are of known provenance (Appendix A). That the specimen from Sabah (Borneo) consistently falls out in a well-supported (except in TvMP) clade with the specimen collected from Sumatra (as well as the third *T. tana* sequence) rather than with the *T. splendidula* specimen from Kalimantan (Borneo) is consistent with the continued recognition of *T. splendidula* as a distinct species, particularly when its poorly supported position is considered. None of the analyses favored a *T. splendidula* + *T. glis* relationship.

The only other nominal species of *Tupaia* whose status has been repeatedly questioned and for which our results offer some illumination (but see below) is *T. palawanensis*, the sole treeshrew occurring on the island of Palawan in the Philippines (Heaney et al., 1998). Corbet and Hill (1992) included *T. palawanensis* in *T. glis*, as did Nowak (1999). The immunological distance-based analyses of Dene et al. (1978, 1980) did not support a close relationship between these two species, and alternative analyses (figured in their 1978 fig. 1) by these authors suggested that *T. palawanensis* may be the most basal of the *Tupaia* species they surveyed. Cluster analysis (UPGMA) of morphometric data from six species of *Tupaia* (including *T. glis* and *T. palawanensis*) and *Urogale* likewise failed to support any close similarity between these taxa (Han et al., 2000). Our analyses similarly found no support for a close relationship between *T. palawanensis* and *T. glis*. Although neither MP, TvMP, nor ML provided strong support for the position of *T. palawanensis*, Bayesian posterior probabilities for a clade consisting of several species of *Tupaia*, including *T. palawanensis* but not *T. glis*, were high (see Section 3). Although such inferences are somewhat limited by our sample, we nonetheless conclude that there is no molecular evidence for a close relationship between *T. glis* and *T. palawanensis*.

Finally, the issue of species limits among *T. glis* and *T. belangeri* deserves mention here. No fewer than 54

published names (species or subspecies) have been synonymized with these two taxa (see Helgen, in press), 27 in each species. A full review of this complicated taxonomic history is well beyond the scope of this paper (see Corbet and Hill, 1992; Dene et al., 1978, 1980; Helgen, in press; Martin, 1990, 2001; Napier and Napier, 1967; Nowak, 1999; Wilson, 1993). Collectively, these species are broadly distributed in southern and SE Asia (Table 1). As currently recognized, *T. belangeri* includes all forms north of a purported contact zone between *T. belangeri* and *T. glis* (Endo et al., 2000) in southern Thailand (Helgen, in press). *Tupaia glis* includes all forms south of this location. Various authors have considered *T. belangeri* to be conspecific with *T. glis* (e.g., Han et al., 2000; Hill, 1960; Napier and Napier, 1967), but immunodiffusion data (Dene et al., 1978, 1980), chromosomal differences (Toder et al., 1992), pelage characters (Endo et al., 2000), and cranial morphometry (Endo et al., 2000) differentiate mainland specimens of *T. glis* from *T. belangeri*. Additionally, Lyon (1913) and Martin (1968) noted differences in mammary formula between *T. belangeri* (3 pairs, with few exceptions) and *T. glis* (2 pairs, with the sole exception of *T. glis discolor*).

Our reasons for highlighting this example involve methodological issues only. At first glance, our sample would appear to be inadequate to address species limits in *T. glis* and *T. belangeri*, given their respective ranges and our relatively small sample sizes. However, we point out that three of these, including two *T. belangeri* and one *T. glis*, are of unknown provenance (see Appendix A). The only one sequenced for this study, FMNH 165412, was obtained from a zoo, and an exhaustive attempt by one of us (L.E.O.) to determine the matrilineal origin of this specimen with respect to collecting locality was unsuccessful. The remaining two sequences appeared in publications focused on interordinal mammalian relationships, and no voucher information was provided by their respective authors. The issue of publishing molecular (or any) data without the corresponding voucher information is a critical one that has been compellingly addressed in this very journal (Ruedas et al., 2000). While locality information may be relatively unimportant for higher-level questions, studies such as this one making subsequent use of these sequences would benefit from having such data. For example, if the previously published sequences from *T. belangeri* and *T. glis* were derived from specimens south and north, respectively, of the currently recognized contact zone between these species, this would shed additional light on the extent of their distributional overlap (or the introgression of mtDNA). But until journals consistently abide by recommended standards for disclosing voucher information (Ruedas et al., 2000; see also J. Mammalogy 82(4) (2001) 1136), this problem is almost guaranteed to persist.

#### 4.4. Biogeographic implications

Treeshrews collectively inhabit one of the most geographically and geologically complex regions of the world (Audley-Charles, 1987; George, 1987; Whitmore, 1987). Their distribution west of Wallace's line (i.e., not extending to Sulawesi [Musser, 1987], Lombok [Kitchener et al., 1990], or islands eastward of these) and their absence on such proximate islands as the Andamans suggests severe limitations to overwater dispersal (with the apparent exception of *Tupaia nicobarica* from the Nicobar Islands, which have never had a landbridge connection to mainland SE Asia or nearby Sumatra [Prashanth and Veenakumari, 1996]). Vicariance can therefore almost certainly be assumed to have played a prominent role in the past diversification and resulting distribution of treeshrews. Although the chronology of island formation is reasonably well understood for greater Sundaland (Voris, 2000; see also Han et al., 2000), the aforementioned lack of resolution among members of the genera *Tupaia* and *Urogale* precludes a rigorous examination of biogeographic patterns at this level with respect to phylogeny. As such, many compelling questions, including the phylogenetic origin of biogeographically enigmatic taxa such as *T. nicobarica*, *T. palawanensis*, *Anathana*, and *Urogale*, and the possibility of a relatively recent adaptive radiation of treeshrews on Borneo (as suggested by Han et al., 2000) remain unanswered. However, we do take issue with the final conclusion reached by Han et al. (2000, p. 12), who invoked short genetic distances between *Urogale* and *Tupaia* in the context of current geographic distributions in their deduction that *Ptilocercus* and *Dendrogale* represent "mainland outgroups" to (presumably) the remaining extant treeshrews. While our analyses support the successively basal divergences of *Dendrogale* and *Ptilocercus*, neither of their distributions can be considered more "mainland" than those of several other treeshrews (e.g., *Tupaia belangeri*, *T. glis*, *T. minor*, and *Anathana*). Indeed, *D. melanura* occurs only in northern Borneo, and *Ptilocercus* occurs throughout much of Borneo and Sumatra, in addition to peninsular Malaysia and Thailand (Table 1). We believe that evidence from the distribution of living scandentians is equally supportive of a Bornean origin of treeshrews, given that six of the currently recognized species are restricted to the island and an additional four species (including *Ptilocercus*, the most basal treeshrew) occur there, as it is to a "mainland" origin (though we note that much of the "mainland" discussed here was insular in the past). Perhaps it is also worth noting that extant dermopterans, the putative sister group of Scandentia (e.g., Murphy et al., 2001a) in Sundatheria, also occur in Borneo, in addition to mainland SE Asia, Sumatra, Java, and the Philippines. On the other hand, a mainland origin may be better supported by fossil evidence. In addition to extant taxa such

as *Anathana*, *T. belangeri*, *D. murina*, *Ptilocercus*, and *Cynocephalus*, several fossil treeshrews have also been found on the mainland (Sargis, 1999, 2004). They have been recovered in the Miocene of Thailand (Mein and Ginsburg, 1997), India (Chopra and Vasishat, 1979; Chopra et al., 1979), Pakistan (Jacobs, 1980), and China (Ni and Qiu, 2002; Qiu, 1986), as well as the Eocene of China (Tong, 1988). In fact, Ni and Qiu (2002) recently claimed that there is fossil evidence of both the *Dendrogale* (i.e., *Prodendrogale*) and ptilocercid lineages at Yuanmou, China, although only teeth have been discovered so far and they have not yet been described or figured (nor have the putative ptilocercid specimens been named). If the two most basally divergent treeshrew lineages were indeed present in the Miocene of southern China, this would make a much stronger case for a mainland origin, especially if the late Eocene *Dermotherium* (Ducrocq et al., 1992) from Thailand is accepted as a dermopteran (see Silcox et al., in press). Only when this fossil evidence is considered does a mainland origin of treeshrews gain support. While the results presented in this paper have shed some light on the phylogenetic history within Scandentia, testing many of the biogeographic hypotheses pertaining to treeshrew evolution must await the accumulation of additional data (Olson et al., in prep.).

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### Appendix A. Voucher specimens

Voucher information for specimens sequenced in this study, arranged alphabetically by genus and species

Species	Source	Tissue type	Locality
<i>Anathana ellioti</i>	FMNH 91265	d	India: Madhya Pradesh
<i>Dendrogale melanura</i>	USNM 292552	d	Malaysia, Borneo: Sabah; Mount Kinabalu, Kamborangah
<i>D. murina</i>	USNM 320779	d	Vietnam: Khanh Hoa; Xuan Phu, 2 km S
<i>Ptilocercus lowii</i>	FMNH 76855	d	Malaysia: Sabah; East Coast Residency, Sandakan Dist, Sapagaya Forest Reserve
<i>Tupaia belangeri</i>	FMNH 165412	f	Zoo animal (ISIS M1194); no locality data available
	USNM 583857	f	Myanmar: Mons State; Kinmun, 5 km NE of, at Yetagon Myaung in Kyaikhtyi Wildlife Sanctuary, 17°26'39"N, 97°05'58"E
	MVZ 186408	f	Vietnam: Vinh Phu Province; Vinh Yen District, Tam Dao; 21°27'14.2"N, 105°38'31"E, 700–1000 m
<i>T. dorsalis</i>	UMMZ 174427	f	Indonesia: West Kalimantan; Ketapang Regency, Gunung Palung National Park, Cabang Panti Research Site. 500–600 m
<i>T. glis</i>	MVZ 192180	f	Indonesia, Sumatra: 3 km NW Bukit Lawang; 450 m
	MVZ 192184	f	Indonesia, Sumatra: Ketambe Research Station, 400 m
<i>T. gracilis</i>	USNZ 109023	f	Malaysia: Sabah; Sepilok
<i>T. javanica</i>	FMNH 47118	d	Indonesia, Java: Gunung Moeria, Keling

(continued on next page)

**Appendix A (continued)**

Species	Source	Tissue type	Locality
<i>T. longipes</i>	JS M02b	f	Malaysia, Borneo: Sabah, Danum Valley Field Center, 4°58'N, 117°48'E, 450–650 m
<i>T. nicobarica</i>	USNM 111753	d	India: Andaman and Nicobar Islands; Little Nicobar
<i>T. palawanensis</i>	FMNH 168969	f	Philippines, Palawan Island: Palawan Province; Puerto Princesa Municipality, Tarabanan River, 150 m
<i>T. splendidula</i>	UMMZ 174429	f	Indonesia, West Kalimantan: Ketapang Regency, Gunung Palung National Park, Cabang Panti Research Site, 1000–1100 m
<i>T. tana</i>	MVZ 192193	f	Indonesia, Sumatra: Ketambe Research Station, 400 m
	JS M11	f	Malaysia, Borneo: Sabah, Danum Valley Field Center, 4°58'N, 117°48'E, 450–650 m
<i>Urogale everetti</i>	FMNH 147781	f	Philippines, Mindanao Island: Bukidnon Province; Mt. Katanglad Range, 18.5 km S, 4 km E Camp Phillips, 2250 m

Museum or zoo catalog numbers are given in the second column (FMNH, Field Museum of Natural History; USNM, United States National Museum of Natural History [Smithsonian Institution]; USNZ, United States National Zoo; MVZ, Museum of Vertebrate Zoology, University of California [Berkeley]; UMMZ, University of Michigan Museum of Zoology; JS, specimens collected by J. South and deposited at the Universiti Malaysia Sabah). Tissue types (f, fresh frozen or buffered tissue; d, dried tissue removed from skull and/or skeleton) and collecting localities (as provided by the loaning institution) are provided in the third and fourth columns, respectively.

**Appendix B. Primers**

Primer (direction) <sup>a</sup>	Sequence (5'–3')	Position <sup>b</sup>
16R1(R)	TACAGAACAGGCTCCTCTAG	606
16R2(R)	AACCAGCTATCACCAGGCTCG	1415
AF1(F)	AAAAGGAGCGGGTATCAAG	181
AF2(F)	TTCGTGCCAGCCACCGCGTACATACGA	327
AF4(F)	CTTAAAGGACTTGGCGGT	592
AF5(F)	CAGTCTATATACCGCCATC	684
AF6(F)	AACGTTAGGTCAAGGTGTA	754
AF7(F)	ACACACCGCCCGTCACCCCTC	912
AR2(R)	CTAATCACCGCTTACGCCG	349
AR3(R)	GCTGAAGATGGCGGTATA	673
AR5(R)	TTACTRCTAAATCCTCCT	832
AR6(R)	TGAAATCTTCTGGGTGTA	1046
C(F)	AAAGCAAARCACTGAAAATG <sup>c</sup>	38
G(R)	TTTCATCTTTTCCTTYCGGTAC <sup>d</sup>	1196
LOF1(F)	AAGGAGGATTTAGYAGTAA	849
LOR1(R)	GCTAGTAGTTCTCTGG	547
LOR2(R)	TGAAGCACCGCCAAGT	583
PtF(F)	AACCACTGACGAAAGTAAC	445
TuF1(F)	CTACACGAYAATTAAGACCCAAACTG	485
TuF2(F)	GAATTAGGCCATAAAGCACG	891
TuF3(F)	TAATCGATAAAACCCCGATA	642
TuF5(F)	GTCATGGGCTACATTTTCT	789
TuF6(F)	GCCCAAGCTGTAATAATGC	412
TuF8(F)	CACGGGTTACAGCAGTGA	242
TuR1(R)	GCAAGACGTTTGTGAGCTAC	193
TuR2(R)	AGCATAGTGGGGTATCTA	490
TuR3(R)	GACTAGAAAATGTAGCCCAT	774
TuR4(R)	GTGTGGTTGAGCAAGACG	204
TuR5(R)	AAGTGCACYTTCCAGTAC	987
TuR7(R)	GCACGTAGTACTCTGGCG	545
TuR8(R)	ACTTAGTCGAACTTTCGTTCA	257
1(F)	AAAAAGCTTCAAACCTGGGATAGATACCCCACTAT <sup>e</sup>	504
2(R)	TGACTGCAGAGGGTGACGGGCGGTGTGT <sup>e</sup>	893

<sup>a</sup> F, forward and R, reverse.

<sup>b</sup> Refers to the position of the 3' end on the mitochondrial genome of *Tupaia belangeri* (GenBank Accession AF217811).

<sup>c</sup> From Springer et al. (1995).

<sup>d</sup> Slightly modified from Springer et al. (1995).

<sup>e</sup> From Kocher et al. (1989).

## Appendix C. Fragments and overlap

Text file listing individual amplification and sequencing products (identified by primer pairs) used to generate continuous 12S sequences used in this study can be found, in the online version, at doi:10.1016/j.ympcv.2005.01.005.

## Appendix D. Annotated alignment

Nexus file containing an annotated alignment of the 12S sequences used in this study can be found, in the online version, at doi:10.1016/j.ympcv.2005.01.005. Extensive comments are provided in brackets; these are viewable in PAUP\* 4.0b10 (Swofford, 2003) but may not be visible when other Nexus-based programs are used (see Dahlberg, 1989).

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